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UNITED STATES ARMY
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ABSTRACT

We reported previously that the protective effect of thiourea and related compounds on UV-irradiated eastern equine encephalitis (EEE) virus and the infectious RNA of this virus appeared to be specific for compounds containing the C=S group, and to be dependent both upon an interaction of the compound with the viral nucleic acid and upon the light-absorbing character of the compound. Further investigation has shown that the effect is more extensive than was reported. Yeast RNA, tested early in this study, was completely without effect. The subsequent finding that compounds not containing a C=S group, e.g., 6-nitro 1 H-benzotriazole, retarded the UV inactivation of EEE virus led to the determination that the purines and pyrimidines, and apparently the nucleosides and nucleotides, all exert a protective effect against UV. Also, compounds that protected EEE virus against UV inactivation were effective as well against ionizing radiation. However, known radioprotective compounds, such as cysteamine, which also protected EEE virus against ionizing radiation, were completely ineffective against UV radiation.

I. INTRODUCTION

In an earlier report,[1] we showed that the inactivation by ultraviolet light of suspensions of partially purified eastern equine encephalitis (EEE) virus and of the infectious RNA of this virus was markedly retarded by the addition of thiourea or of compounds related to thiourea. We reported that the effect appeared to be specific for compounds containing the double-bonded carbon-sulfur group. Also, the phenomenon appeared to depend upon an interaction of the additive with the viral nucleic acid as well as upon capacity of the compound to absorb light in the ultraviolet region.

Further investigations indicate that the effect is more extensive than was previously reported in that it is not restricted to compounds containing the C=S group and applies also to ionizing radiation. Details of these investigations are presented in this report.

II. MATERIALS AND METHODS

The virus used in all tests was the Louisiana strain of EEE virus.[2] It was propagated in Maitland-type chick embryo culture and partially purified by one cycle of differential centrifugation. Sediments of the high-speed centrifugation were resuspended in phosphate buffer, pH 7.8. Virus in this partially purified condition was used in all tests.

A volume of 1.0 ml of the virus suspension was mixed in a small petri dish with an equal volume of a phosphate buffer solution of the compound being tested. Control samples were prepared in the same manner by mixing equal volumes of virus suspension and buffer. Samples were irradiated with constant agitation under a 15-watt germicidal lamp at a distance of 20 cm. The lamp intensity was 520 microwatts per square cm at this distance. At specified intervals portions of the samples were withdrawn and assayed for infectivity in embryonated eggs.

For ionizing radiation experiments virus suspensions of 1.0 ml volume, mixed with equal volumes of the compound or of buffer, were frozen in sealed glass ampoules and exposed in the frozen state to gamma radiation from a cobalt-60 source at a rate of 125,000 roentgens per minute. These samples remained frozen until time of assay.

III. RESULTS

A. COMPARISON OF PROTECTIVE EFFECTS OF THIOUREA AND 2-BUTANONE THIOSEMICARBAZONE

In screening compounds for their effect on the UV inactivation of EEE virus, substituted thioureas (such as phenylthiourea) and compounds having structures similar to, but more complex, than thiourea (such as diethyldithiocarbamate) were more effective than thiourea itself when compared at concentrations within the limits of solubility of the less soluble compounds. One of the most effective compounds tested to date is the thiosemicarbazone of ethyl methyl ketone, or 2-butanone thiosemicarbazone. A comparison of the protective effect of this compound with that of thiourea is presented in Figure 1. Although the greatest protection was given by 0.5 M thiourea, note that 0.01 M 2-butanone thiosemicarbazone was much more effective than 0.01 M thiourea and almost as effective as 0.1 M thiourea.

B. PROTECTIVE EFFECT OF COMPOUNDS NOT CONTAINING THE CARBON-SULFUR DOUBLE-BOND

Continued screening of compounds has shown that the presence of a C=S bond in the molecule is not a requirement for a compound to be protective. The first nonsulfur compound found to have activity was 6-nitro 1 H-benzotriazole. As shown in Figure 1, this compound, at a concentration of 0.01 M, was more effective than 0.01 M thiourea but considerably less protective than butanone thiosemicarbazone. Another nonsulfur compound, dipyridyl, also showed protective activity, but much less than thiourea.

Early in these studies, yeast RNA had been tested at UV absorbancy levels comparable to those of thiourea and determined to be without effect. Later, when thiouracil was tested in parallel with uracil, it was found that not only the sulfur analog, but uracil itself, had a strong protective effect on UV-irradiated EEE virus. Other pyrimidines, as well as purines, nucleosides and nucleotides, were tested, and all protected against UV radiation. Table 1 indicates the results obtained with representative compounds of this group. About the same degree of protection was provided by all of these compounds. We decided that a comparison of the effectiveness of polyadenylic acid and adenylic acid might indicate whether the size of the molecule was important in the mechanism of protection. As shown in Figure 2, adenylic acid was much more effective than polyadenylic, suggesting that the large molecule might be unable to interact with the viral nucleic acid. RNA at this concentration was also ineffective.

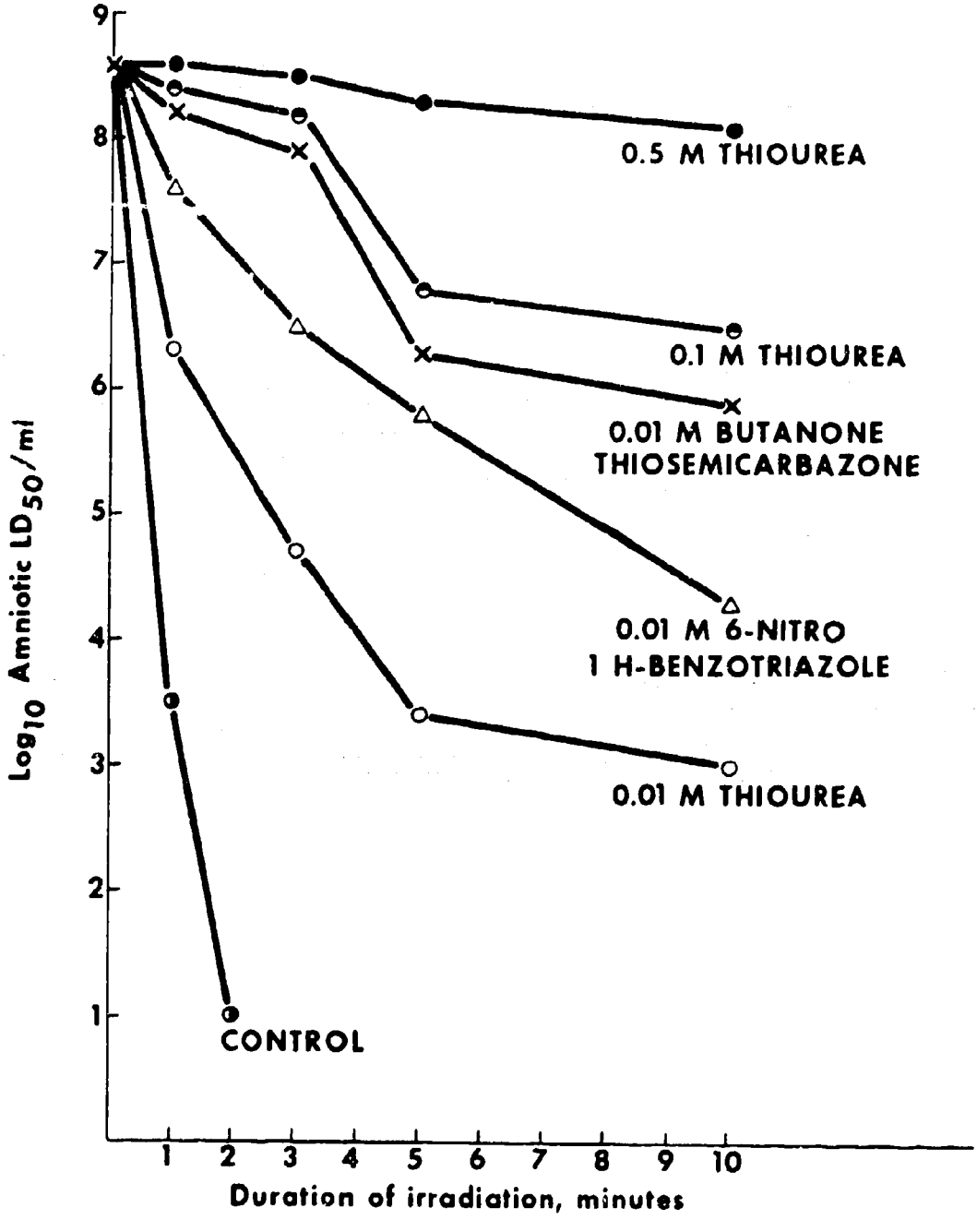

Figure 1. Protection of Eastern Equine Encephalitis Virus Against Inactivation by UV Radiation.

TABLE 1. EFFECT OF REPRESENTATIVE COMPOUNDS OF THE PURINE-PYRIMIDINE TYPE ON UV-IRRADIATED EEE VIRUS

Compound Added[a]	Infectivity for Embryonated Eggs[b] Duration of Irradiation, minutes			
	0	1	3	5
None	8.5	3.4	1.0	0
Uracil	8.5	7.4	6.3	5.5
Adenosine	8.3	7.6	7.1	5.3
Cytidylic acid	8.5	7.8	6.0	5.4
Thymine	8.6	7.9	6.0	4.1
Azathymine	8.6	6.8	5.3	4.1

a. Concentration 0.01 M.
b. Log_{10} amniotic LD_{50} per ml.

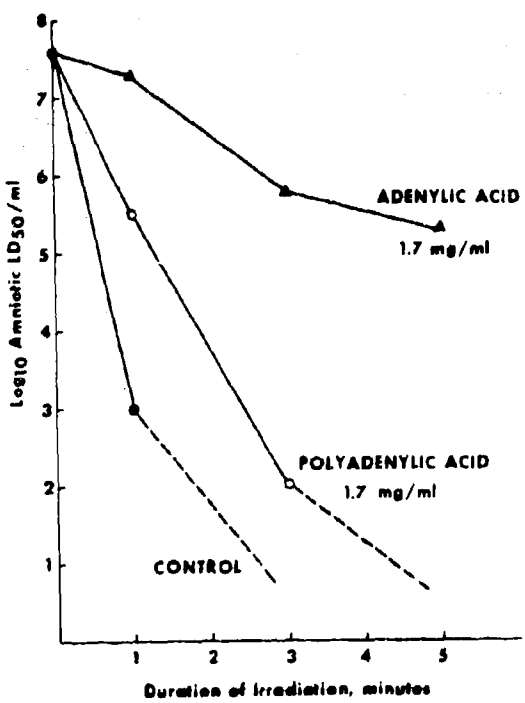

Figure 2. Comparison of Adenylic and Polyadenylic Acids for Effect on UV-Irradiated EEE Virus.

C. PROTECTION OF EEE VIRUS AGAINST INACTIVATION BY IONIZING RADIATION

We were interested in determining whether thiourea and other compounds that protected EEE virus against UV inactivation would have any effect on the inactivation of virus by ionizing radiation. In initial experiments, thiourea was compared with cysteamine, a sulfhydryl compound that has been widely employed as a radioprotective compound, for their relative protective effects against inactivation of the virus by gamma radiation. Virus samples in the presence of 0.01 M and 0.1 M concentrations of thiourea or cysteamine, together with control samples, were exposed in the frozen state to doses of 500,000, 1 million, and 2 million roentgens. As shown in Figure 3, thiourea did retard the inactivation of the virus; the 0.01 M concentration was more effective than the 0.1 M at doses of 500,000 and 1 million roentgens. Although 0.01 M cysteamine produced greater protection than thiourea, 0.1 M cysteamine was detrimental to viral infectivity (in the absence of any radiation) and did not appear to provide any significant protection against ionizing radiation. The control virus, i.e., partially purified virus in phosphate buffer, showed a straight-line inactivation curve indicative of a single-hit mechanism of inactivation. In the presence of cysteamine or thiourea the virus displayed a more complex type of inactivation curve, suggesting a delay in the effect of the radiation. After being exposed to 2 million roentgens, virus suspensions containing these compounds still possessed infectivity titers between $10^{3.7}$ and $10^{4.7}$ LD_{50} per ml. Notice that the inactivation curves for samples containing cysteamine and thiourea were similar in shape, indicating perhaps that these compounds protect by the same mechanism. The two nonsulfur compounds, nitrobenzotriazole and dipyridyl, gave significant protection against ionizing radiation. However, several compounds of the purine-pyrimidine type that we have tested have provided no protection.

Data in Table 2 indicate that radioprotective compounds of the cysteamine type, which (as shown in Figure 2) were effective against ionizing radiation, were completely ineffective against UV radiation.

In summary, compounds tested fall into three groups: (i) compounds of the carbon-sulfur double-bond type and, in addition, 6-nitrobenzotriazole and dipyridyl, protect against both UV and ionizing radiation; (ii) radioprotective compounds of the cysteamine type protect virus against ionizing radiation but not against UV radiation; and (iii) compounds of the purine-pyrimidine type protect virus against UV but not against ionizing radiation.

TABLE 2. COMPARISON OF COMPOUNDS WITH DIFFERENT SULFUR GROUPS FOR PROTECTION AGAINST ULTRAVIOLET INACTIVATION OF EEE VIRUS

Compound Added	Infectivity for Embryonated Eggs[a,b] After 60 seconds' exposure
None	1.0
Cysteine, 0.1 M	<1.0
Cysteamine, 0.01 M	<3.0
Cystamine, 0.01 M	<3.0
Thiourea, 0.01 M	6.1
Thiourea, 0.1 M	7.3

a. Log_{10} amniotic LD_{50} per ml.
b. Titer before exposure $10^{7.6}$ LD_{50} per ml.

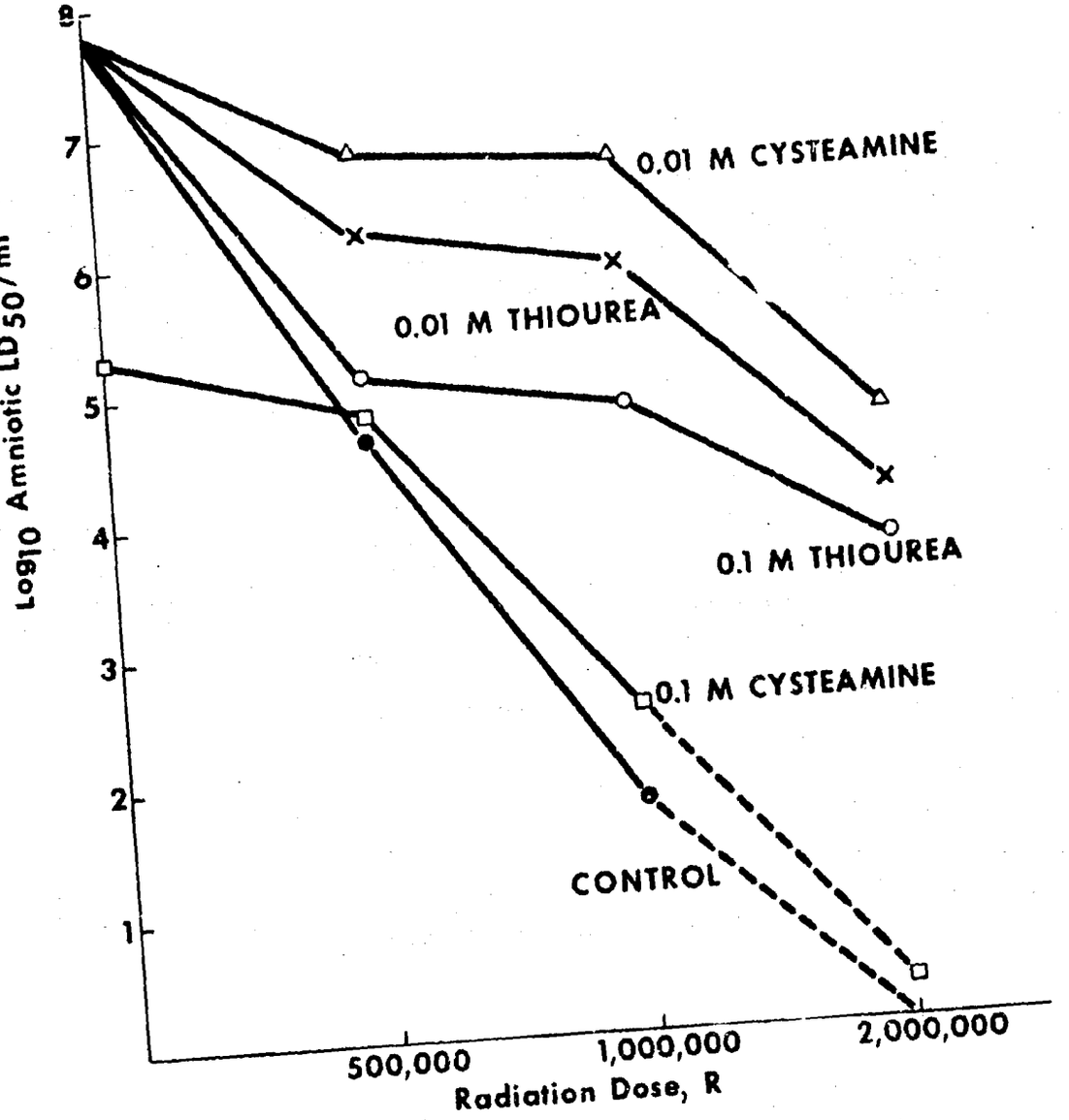

Figure 3. Comparative Protective Effects of Thiourea and Cysteamine Against Inactivation of Eastern Equine Encephalitis Virus by Ionizing Radiation.

IV. DISCUSSION

From a consideration of several kinds of evidence, it appears that the protection of EEE virus against inactivation by UV radiation observed for thiourea (and probably for other protective compounds) is associated with a direct interaction of the compound with the nucleic acid of the virus, and is not merely the result of the compound acting as a filter to remove some of the radiation. This conclusion is based on the following observations: (i) thiourea protects the isolated infectious RNA of EEE virus against rapid inactivation by UV; (ii) a number of compounds that absorb strongly in the ultraviolet region, e.g., benzimidazole and phenylalanine, afford no protection; (iii) solutions of protective compounds used as filters above the virus suspension do not protect; (iv) polyadenylic acid is not effective, in contrast to the smaller molecule, adenylic acid; and (v) the elution pattern of infectious RNA from methylated bovine albumin is changed markedly by thiourea. Moreover, in considering compounds that are protective, it appears that strong absorbance in the UV region per se does not correlate with the degree of protection provided by the compound. For example, dipyridyl absorbs more strongly at all wavelengths in the region of 230 to 300 mμ than does 2-butanone thiosemicarbazone, yet it is much less effective in protecting against UV.

A clue to the mechanism of protection might be found in the suggestion of Sadler[3] who, in reference to the thiosemicarbazones that have antiviral activity, such as isatin-β-thiosemicarbazone, stated that "these planar molecules, with their hydrogen-bonding propensities, interact directly and specifically with virus nucleic acid intracellularly, without impairing normal cellular function." Sadler refers to the work of Lerman[4] with the acridine derivatives and DNA. Lerman suggested that the planar configuration of the acridines and the probability of strong electronic interactions favor their flat, face-to-face binding to the bases of the nucleotides. It was postulated that untwisting of the double helix provides sufficient space for the insertion of an acridine molecule, leaving undisturbed the hydrogen-bonded pairing of the nucleotides constituting each layer. With information derived from viscosity measurements on DNA-acridine complexes and the X-ray diffraction pattern given by the complex of DNA with proflavine, Lerman concluded that of the possible ways to bind acridines to DNA, only intercalation into the helix by extension of the "backbone" was compatible with the available evidence. Sadler points out that even stronger H-bonding characteristics occur in the planar tricyclic isatin-β-thiosemicarbazone molecule, and none of the evidence to date eliminates intercalation with DNA as a mode of action.

The compounds that we have found to be effective in protecting EEE virus against UV inactivation have structural characteristics related to the thiosemicarbazone molecule, to the pyrimidine-purine rings, or, in the case of dipyridyl, to the acridine molecule. By analogy with the conclusions of Sadler and Lerman, we might expect that these compounds bind directly to viral nucleic acid, recognizing that intercalation of the protective compound between the bases of the nuclectides is a possible mode of action. Beukers[6] reported recently that thymine dimer formation, generally accepted as one of the primary inactivating effects of UV irradiation of DNA, was completely prevented when proflavine, an intercalating dye, was added prior to irradiation. If proflavine molecules are intercalated between neighboring thymine molecules, thymine dimer formation is not possible.

Beukers' results raise the question as to whether the protection against UV inactivation that we have observed is related to the prevention or retardation of uracil dimer formation in the viral RNA by a mechanism of intercalation of the molecules of the additive between the bases of the nucleic acid. In any case, since compounds that are effective all absorb in the ultraviolet region, their direct contact with the nucleic acid would be expected to modify the absorbancy of the nucleic acid, and thus modify also the rate of loss of infectivity by the virus.

LITERATURE CITED

1. Wachter, R.F.; Comer, J.F. 1965. Protective effect of thiourea on ultraviolet-irradiated eastern equine encephalitis virus. Bacteriol. Proc. p. 109.

2. Howitt, B.F.; Bishop, L.K.; Gorrie, R.H.; Kissling, R.E.; Hauser, G.H.; Trueting, W.L. 1948. An outbreak of equine encephalomyelitis, eastern type, in southwestern Louisiana. Proc. Soc. Exp. Biol. Med. 68:70-72.

3. Sadler, P.W. 1965. Antiviral chemotherapy with isatin-β-thiosemicarbazone and its derivatives. Ann. N.Y. Acad. Sci. 130:71-79.

4. Lerman, L.S. 1961. Structural considerations in the interaction of DNA and acridines. J. Mol. Biol. 3:18.

5. Beukers, R. 1965. The effect of proflavine on UV-induced dimerization of thymine in DNA. Photochem. Photobiol. 4:935.

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